RESEARCH ARTICLE

Macerated-Pineapple Core Crude Extract-derived Bromelain Has Low Cytotoxic Effect in NIH-3T3 Fibroblast

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Abstract

BACKGROUND: Bromelain is a sulfhydryl proteolytic enzyme that can hydrolyze protein, protease or peptide. Bromelain can be found in pineapple stem, fruit and core. Bromelain is composed of 212 amino acid residues with cysteine-25 forming a polypeptide chain that can hydrolyze peptide bonds by H_2O. In medicine, bromelain has been developed as antibiotic, cancer drug, anti-inflammatory agent and immunomodulator. In dentistry, bromelain has potential to reduce plaque formation on the teeth and to irigate root canal.

METHODS: Pineapple core was dried for 3 days to get simplicia. Then simplicia was extracted with water solvent for 24 hours. After that, the macerated-pineapple core crude extract-derived bromelain (PCB) was separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue (CBB) staining to ensure the presence of bromelain. In cytotoxic test, NIH-3T3 fibroblast cultures were treated with extracts in various concentrations to for 24 or 48 hours. Number of fibroblasts was calculated using 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay.

RESULTS: Pineapple core extraction using maceration method produced relative high yield (concentration: 1.5424 g/mL) of bromelain, which was confirmed by CBB staining results with the molecular weight of 33 kDa. Based on cytotoxic test results of PCB on NIH-3T3 fibroblasts, 24-hours-incubation LD50 was 95.7 g/L, while 48-hours-incubation LD50 was 51.1 g/L.

CONCLUSION: PCB has low cytotoxic effect in NIH-3T3 fibroblasts.

KEYWORDS: bromelain, pineapple, extract, cytotoxic, MTT

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Introduction

Bromelain can be found in various parts of pineapple, including stem, leaf, crown, peel, fruit and core.(1) However, the core yielded highest bromelain activity recovery of 106% and 5.2 purification fold.(1) Beneficial effects of bromelain are related to its multiple constituents. (2) Although primarily comprised of sulfhydryl-containing proteolytic enzymes, bromelain also contains escharase (2), peroxidase, acid phosphatase, glucosidases, cellulases, several protease inhibitors, glycoproteins, carbohydrates, and organically bound calcium.(3) Bromelain is made up of 212 amino acids and the molecular weight is 33 kDa. (4) Bromeline is also composed of several distinct cysteine proteolytic fractions ranging in size from 15 to 27 kDa.(5)

Bromelain was widely reported to have various active molecular properties. It can activate various innate immune cells including macrophages, dendritic cells, natural killer cells and $CD4^+$ T cells.(6-9) It can also induce apoptosis in skin tumors, breast cancer cells and gastrointestinal carcinoma cells (2,10) and inhibit L-1 cells tumor growth. (11) In addition, bromelain can inhibit human cytochrome P450 2C9 activity.(12) In apoptosis induction, bromelain was reported to activate caspase cascades, cleavage of Poly Adenosine Diphosphate (ADP) Ribose Polymerase (PARP) and p53, overexpression of cytochrome C, attenuation of phosphorylated Akt and B Cell Lymphoma 2 (Bcl2), and removal of Mucin 1 (MUC1).(2) In contrary, bromelain was reported to have anti-apoptosis activity by increasing phosphorylation of Akt and Forkhead Homeobox type O (FOXO) 3A.(5) For anti-tumor and anti-inflammatory, Extracellular Signal-regulated Kinase 2 (ERK-2) Mitogen-activated Protein Kinases (MAPK) and p21ras was disrupted by of bromelain.(13)

Bromelain are considered to have a range of beneficial properties as anti-inflammatory, analgesic actions, anti-oedematous, anti-thrombotic, anti-diarrheal, antibiotics, anti-tumor, skin debridement, digestive aid, musculoskeletal injuries, reduces the blood pressure level, prevents aggregation of blood platelets, activates plasmin, supports the oxidative burst and fibrinolytic effects.(14-17) In humans, bromelain has been well documented to increase blood and urine levels of antibiotics and results in higher blood and tissue levels of tetracycline and amoxicillin when they are administered concurrently with bromelain. (18) Bromelain has been successfully used as a digestive enzyme because of its wide pH range, bromelain has activity in the stomach as well as the small intestine.(19) In skin debridement, the use of topical bromelain for frostbite eschar removal was investigated, after two topical applications of bromelain, frostbite injuries remained un-affected.(20) In dentistry, bromelain exerts an anti-bacterial effect against potent periodontal pathogens, including *Streptococcus mutans*, *Enterococcus faecalis*, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. (21) Since bromelain has many useful properties, we would like to know the safety of bromelain usage. For this purpose, we currently performed pineapple core crude extract and cytotoxic test on NIH3T3 fibroblast.

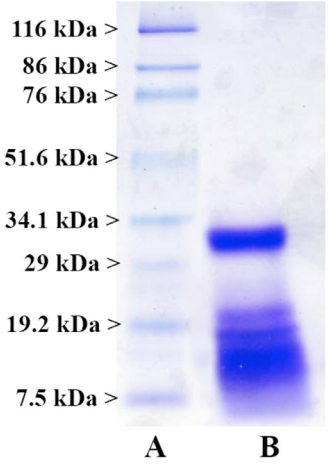

Figure 1. CBB Staining of PCB. Macerated-pineapple core crude extract was separated with SDS-PAGE followed by CBB staining. SDS-PAGE and CBB staining were carried out at described in the Methods. **A**: size marker; **B**: PCB.

Methods

Extraction of Bromelain from Pineapple Core

Pineapples were collected from Bogor, Indonesia. Pineapples were peeled and its cores were collected. Pineapple cores were dried at 40°C for 3 days, weighed and milled. Extraction was performed using maceration method with water solvent in ratio of 1:10 for 24 hours at 4°C. The solvent was added 3 times in 24 hours, then filtered and evaporated with a rotary evaporator (Buchi Rotavapor R-124, Buchi, Flawil, Switzerland) at 40°C. Maceration results were stored in a refrigerator at 4°C.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue (CBB) Staining

Macerated-pineapple core crude extract was mixed with loading buffer in a ratio of 4:1 and boiled for 1 minute. Ten µl of boiled mixtures were then separated by SDS-PAGE followed by CBB staining for 30 minutes, to ensure the presence of bromelain.

NIH-3T3 Fibroblast Culture

NIH-3T3 fibroblasts were cultured using Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B in a humidified, 37°C, 5% CO_2 incubator. Upon reaching 80% confluency, fibroblasts were subcultured and propagated.

Cytotoxic Test

Cytotoxic test was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay (Sigma) as described previously.(22,23) The MTT assay provides a quantitative measurement of viable fibroblasts by determining the amount of formazan crystals produced by metabolically active fibroblasts. Briefly, $1x10^4$ fibroblasts were seeded into each well of 96-well plates in medium containing active agent, 1:1,000 diluted hydrogen peroxide or medium merely. Fibroblasts were incubated for 24

or 48 hours. Ten µl of 5 mg/ml MTT in phosphate buffer saline (PBS) was added to each well. The plate was then incubated for 4 hours, and then the medium was discarded and formazan crystals were dissolved in 100 µl of 0.1N HCl. The absorbance of fibroblasts was measured at 570 nm by a microplate reader. Untreated fibroblasts were counted with a hemacytometer and used for interpolating the absorbance.

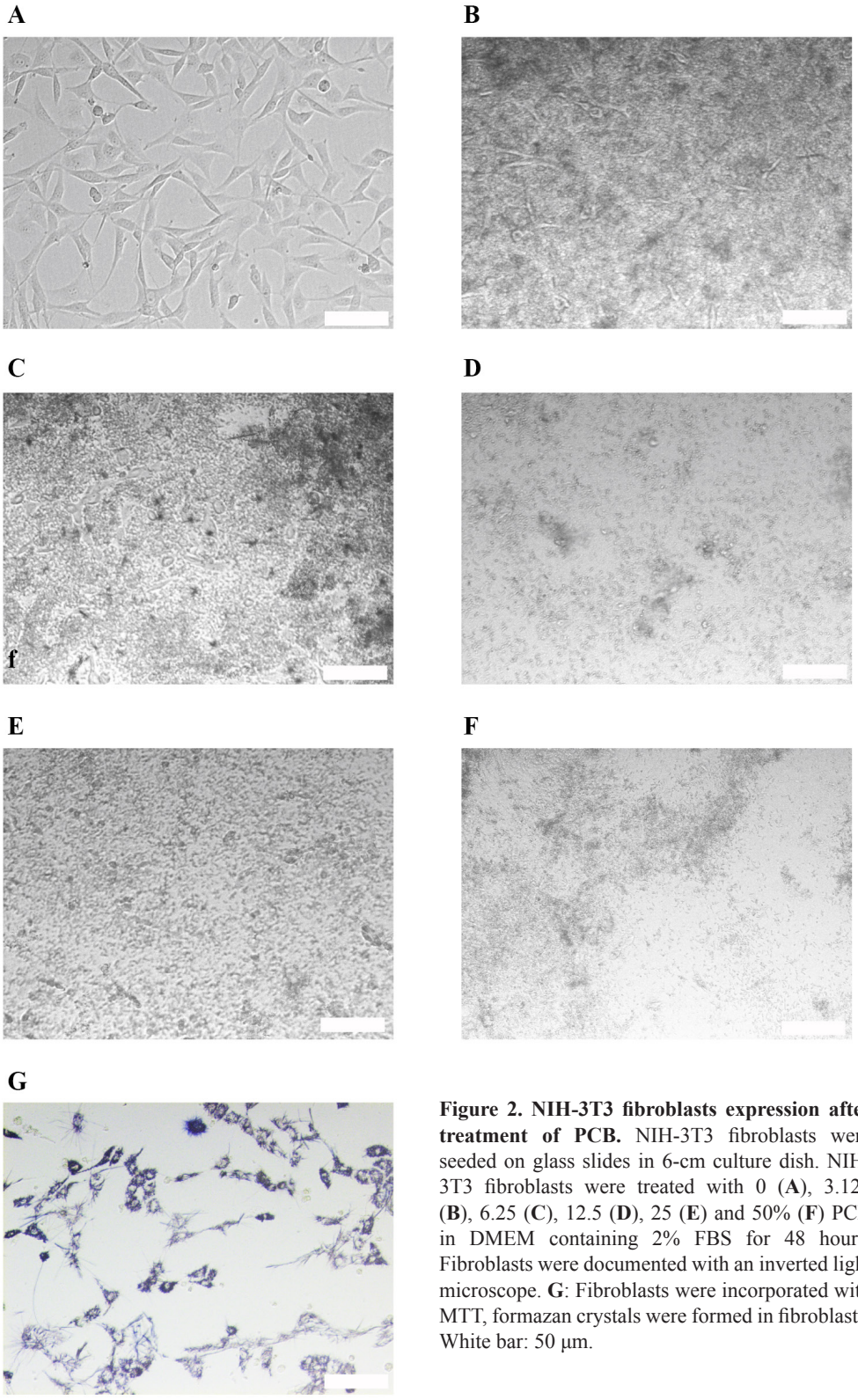

Figure 2. NIH-3T3 fibroblasts expression after treatment of PCB. NIH-3T3 fibroblasts were seeded on glass slides in 6-cm culture dish. NIH-3T3 fibroblasts were treated with 0 (**A**), 3.125 (**B**), 6.25 (**C**), 12.5 (**D**), 25 (**E**) and 50% (**F**) PCB in DMEM containing 2% FBS for 48 hours. Fibroblasts were documented with an inverted light microscope. **G**: Fibroblasts were incorporated with MTT, formazan crystals were formed in fibroblasts. White bar: 50 µm.

Results

Macerated-Pineapple Core Crude Extract-derived Bromelain (PCB)

One hundred pineapples weighed 30 kg, were collected. After skin peeling and fruit removal, 7 kg of core were obtained. After drying, 700 g of simplicia was obtained. After maceration with water solvent and evaporation, 131.1 g of crude extract with total volume of 85 mL (concentration: 1.5424 g/mL), was obtained. The extract was then diluted 1:10 for further purposes. Later on, macerated-pineapple core crude extract was examined for its content. We found that 33 kDa of bromelain in relative high yield contained in the extract, which was indicated by SDS-PAGE followed by CBB staining (Figure 1). Several possible cysteine proteolytic fractions of bromelain near to 19.2 kDa were observed as well.

Effect of PCB on NIH-3T3 Fibroblasts

Our results showed that by addition of PCB in NIH-3T3 fibroblast culture, number of NIH-3T3 fibroblasts were less when treated with higher concentration of bromelain (Figure 2). Highest fibroblast number was observed in 0% PCB, followed by 3.125% PCB, 6.25% PCB, 12.5% PCB, 25% PCB and 50% PCB respectively. By counting the fibroblast number with MTT assay for 24 hours, we found that the number for the group treated with 0% PCB was 11,088, while 3.125% PCB was 10,562; 6.25% PCB was 9,953; 12.5% PCB was 9,621; 25% PCB was 7,711; 50% PCB was 6,992 (Figure 3A&B). Meanwhile, for fibroblast number with MTT assay for 48 hours, we found that the number for the group treated with 0% PCB was 13,087, while 3.125% PCB was 10,179; 6.25% PCB was 9,396; 12.5% PCB was 8,650; 25% PCB was 6,600; 50% was 5,928 (Figure 4A&B).

LD_{50} of PCB on NIH-3T3 Fibroblasts

Based on data from Figure 3, LD_{50} in 24 hours was calculated. Percentage of PCB was converted to percentage of medium. Then graph of percentage of medium against number of fibroblast was made. A median line of y=82.248x+2,425.5 was obtained, y=number of fibroblast and x=percentage of medium. Therefore LD_{50}=100-x. We found that 24-hours LD_{50} of PCB was 62.05%. This equaled to 62.05% x 1.5424 g/mL x 1/10 = 0.0957 g/mL or 95.7 g/L. Meanwhile LD_{50} in 48 hours was calculated based on data Figure 4. Similar calculation method was performed. A median line of y=126.25x-1,471.6 was obtained, y=number of fibroblast and x=percentage of medium. Since LD_{50}=100-x, we found that 48-hours LD_{50} of PCB was 33.15%. This equaled to 33.15% x 1.5424 g/mL x 1/10 = 0.0511 g/mL or 51.1 g/L.

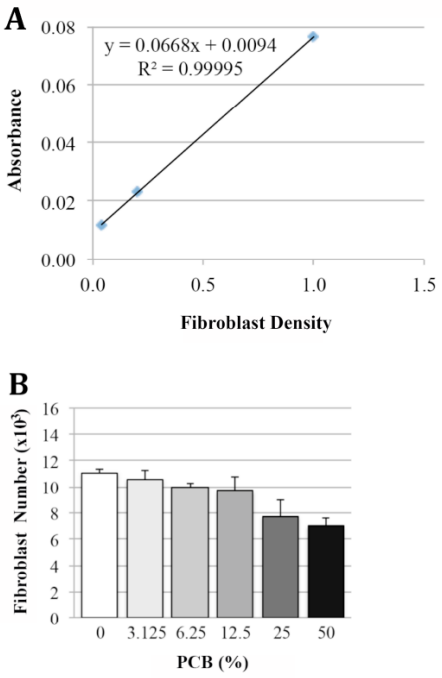

Figure 3. Effect of PCB on viability of NIH-3T3 fibroblasts for 24 hours. Ten thousand NIH-3T3 fibroblasts were seeded into each well of 96-well plates. NIH-3T3 fibroblasts were treated with 0, 3.125, 6.25, 12.5, 25 and 50% PCB in DMEM containing 2% FBS for 24 hours. Fibroblast viabilities were measured with MTT assay as described in Methods. **A**: Obtained absorbances were correlated with fibroblast density (1.0 fibroblast density equaled to 11,094 fibroblasts). **B**: Interpolated number of viable NIH-3T3 fibroblasts after treated with PCB in various concentrations for 24 hours.

Discussion

We successfully produce high yield PCB with confirmed molecular weight. When PCB was added to NIH-3T3 fibroblast cultures for 24 hours, number of NIH-3T3 fibroblast was decreased slightly upon increasing PCB concentrations. Meanwhile additional decrease in number of NIH-3T3 fibroblast was obtained when the NIH-3T3 fibroblast culture was treated for 48 hours. After calculation, we found that 24-hours-incubation LD50 of PCB was 95.7 g/L and 48-hours-incubation LD50 of PCB was 51.1 g/L.

Our results is in accordance with the studies reported for bromelain, which was considered to have very low toxicity.(24) It has been reported that LD50 was greater than 10 g/kg.(24) Moreover, in vivo toxic test on dogs, with increasing levels of bromelain up to 750 mg/kg administered daily, showed no toxic effects after six months.(24) Dosages of 1.5 g/kg/day administrated to rats showed no carcinogenic

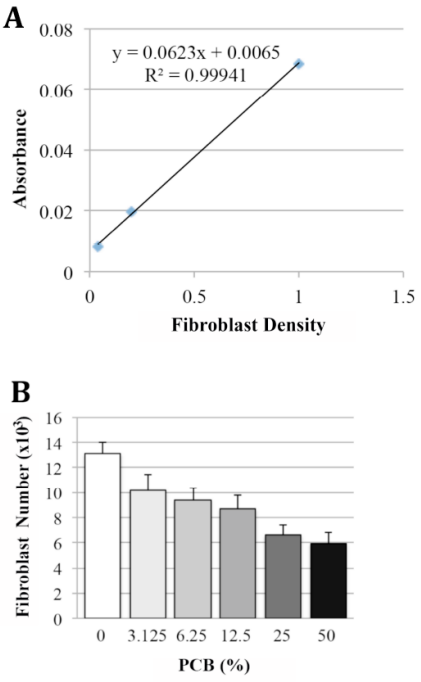

Figure 4. Effect of PCB on viability of NIH-3T3 fibroblasts for 48 hours. Ten thousand NIH-3T3 fibroblasts were seeded into each well of 96-well plates. NIH-3T3 fibroblasts were treated with 0, 3.125, 6.25, 12.5, 25 and 50% PCB in DMEM containing 2% FBS for 48 hours. Fibroblast viabilities were measured with MTT assay as described in Methods. **A**: Obtained absorbances were correlated with fibroblast density (1.0 fibroblast density equaled to 13,938 fibroblasts). **B**: Interpolated number of viable NIH-3T3 fibroblasts after treated with PCB in various concentrations for 48 hours.

or teratogenic effects.(24) In human clinical test, side effects are generally not observed, however caution is advised if administering bromelain to individuals with hypertension since one report indicated individuals with pre-existing hypertension might experience tachycardia following high doses of bromelain.(25) Other experiments have previously shown that bromelain at high concentrations does not adversely affect cell viability, similarly, bromelain concentration of 50 mg/ml had no effect on the viability of chondrocytes.(26) Other experiments have previously shown that bromelain proteolytically removes certain cell surface molecules by cleavage of the peptide bond. (13,27,28)

PCB has low cytotoxic effect in NIH-3T3 fibroblasts, meanwhile there are many potential therapeutic benefits of bromelain, For example, bromelain exerts an anti-bacterial effect against potent periodontal pathogens, including *Streptococcus mutans* and *Porphyromonas gingivalis*.(21) Therefore, further studies in bacteria elimination should be pursued, whereas PCB could be useful to disrupt dental plaque formation disturbance and to irrigate root canal.

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